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## CHEMICAL BIOLOGICAL CENTER

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# 20-L SCALE-UP FERMENTATION AND BIOTRANSFORMATION OF GASEOUS PROPANE TO PROPANOL

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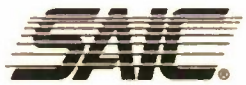


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Two *E. coli* BW28357 hosts (one with pyk A mutation and one without) harboring the same PMO plasmid were used in a 20-L scale-up fermentation process to compare the effect of pyk A mutation in propane to propanol biotransformation yield. Also, one additional 20-L fermentation run with *E. coli* BW28357 without pyk A mutation was conducted without IPTG induction to serve as a baseline in terms of cell yield and dissolved oxygen profile. The same media and operating conditions were used for each three runs. For the two IPTG induced runs, the same timing (around 4.5 hr into fermentation w/optical density of approximately 0.8) was used. No significant differences in dissolved oxygen profiles and final optical density in each fermentation run (approximately 10) were observed. The levels of expressed PM-450 were significant and similar at approximately 30% of the total cellular proteins. However, low propanol yields of approximately 2 mM for both fermentation runs, which represented only 0.0017% of the total gaseous propane fed during the course of each fermentation run, indicated a low catalytic turnover rate of the expressed P450 BM-3 enzymes.

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## 1. INTRODUCTION

This study was a part of a collaborative effort by Science Applications International Corporation (SAIC), California Institute of Technology (Caltech), and U.S. Army Edgewood Chemical Biological Center (ECBC) on a 6.2 Institute for Collaborative Biotechnologies (ICB) applied research project entitled "Methanol: Bioprocess for Alternative Fuel from Wastes and Stranded Methane Reserves." Research at Caltech on the directed evolution of cytochrome P-450 BM-3, originally from *Bacillus megaterium,* to accept and catalyze progressively smaller straight-chain alkane molecules (i.e., ultimately methane) resulted in a PMO plasmid that can readily be transformed into a standard bacterial host such as *Escherichia coli.* The expressed P-450 BM-3 protein oxidizes either propane or ethane to its respective alcohol (Meinhold et al., 2005; Fasan et al., 2007). However, the expressed P-450 BM-3 protein is specifically NAD(P)H-dependent as its cofactor.

The native *E. coli* metabolic pathway has several reactions that consume NAD(P)H/NADH reducing equivalents (e.g., production of acetate, ethanol, lactate, ATP [from oxidative phosphorylation] and formate) and limit the NAD(P)H/NADH pool that could be re-channeled to the P-450 BM-3 catalytic reaction. Hence, we have generated a single gene knockout mutant of an *E. coli* that blocks one of these usages of reducing equivalents. The objective of this report is to summarize the results from a 20-L scale-up fermentation and present data on the propane to propanol biotransformation process by two *E. coli* hosts harboring the same PMO plasmid, one without and one with pyruvate kinase A (pyk A) mutation.

## 2. MATERIALS AND METHOD

*E. coli* BW28357 host and Δ pyk A mutant *E. coli* harboring the same PMO plasmid were provided by Dr. Frances Arnold (Caltech) and Dr. Ilona Fry (SAIC), respectively.

The 20-L fermentation media consisted of the following: 0.5% w/v glucose; 10 g/L yeast extract; 0.1 mM $CaCl_2$; 1 mM $MgSO_4$; 0.01 mM ferric citrate; M9 salts (42 mM $Na_2HPO_4$; 22 mM $KH_2PO_4$; 8.6 mM NaCl; and 37mM $NH_4Cl$); trace metals (50 µM $FeCl_3$; 20 µM $CaCl_2$; 10 µM $MnSO_4$; 10 µM $ZnSO_4$; 2 µM $CoSO_4$; 2 µM $CuCl_2$; 2 µM $NiCl_2$; 2 µM $Na_2MoO_4$; and 2 µM $H_3BO_3$), and 50 mg/L sodium ampicillin, as provided by Mike Chen (Caltech).

The 20-L fermentation was conducted using a Micros 30 (New Brunswick Scientific, Edison, NJ) bioreactor under the following operating conditions: 30 °C temperature, 300 rpm mixing speed, 20 standard liters per minute (slpm) air flow,

1 psi overhead pressure, and pH 7 (controlled with automatic addition of either 3M $H_3PO_4$ or 3M NaOH) for cultivation and expression. The expression of P450 BM-3 was induced by adding 0.25 mM IPTG and 0.25 mM d-aminolevulinic acid when the optical density (OD) at 600 nm (Genesys 20, ThermoSpectronic) inside the 20-L fermentor reached around 0.8 (except for the baseline 20-L fermentation run). When the cultures reached an OD value of approximately five, the air flow was reduced to 10 slpm, and the propane was introduced at a 10 slpm flow for the biotransformation of propane to propanol.

The concentration of propanol in culture media was monitored by Hewlett-Packard Gas Chromatography 5890 Series II equipped with Stabilwax® w/Integra-Guard column (30 m, 0.32 mm ID, 1 μm film thickness), and HP 7673 autosampler.

Relative quantification of expressed P450 BM-3 proteins in samples was achieved by running a 0.8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, MiniProtein II, BioRad) at a constant voltage of 150 V for 45 min, then analyzing bands on a Molecular Imaging System (Imaging Station 4000R, Kodak).

## 3. RESULTS AND DISCUSSION

OD and dissolved oxygen (DO) profiles during growth of *E. coli* BW28357 and Δ pyk A mutant *E. coli* harboring the PMO plasmid with IPTG induction were compared to the baseline OD and DO profiles during growth of *E. coli* BW28357 without the induction (Figure 1). The OD and DO profiles of the baseline *E. coli* BW28357, without the IPTG induction, served as a basis for the subsequent timings of IPTG and propane inductions in *E. coli* BW28357 and Δ pyk A mutant *E. coli* 20-L fermentation runs.

The longer run times in *E. coli* BW28357 and Δ pyk A mutant with the IPTG induction also represented the propane-to-propanol biotransformation phase, which is absent in the baseline *E. coli* BW28357 without the IPTG induction.
For the biotransformation phases, in which DO is required for the expressed P450 BM-3 activities in transforming propane to propanol, DO tension was maintained above 40%.

Similar OD profiles (i.e., both in rates and final magnitudes of ODs, approximately 10) in all the three fermentation runs indicated that there was practically insignificant metabolic energy demand by the expression of P450 BM-3 proteins. Otherwise, due to metabolic energy siphoned off for the expression of P450 BM-3 proteins, the rates of OD increases and magnitudes of final ODs would have been less than those of the baseline *E. coli* BW28357 without the IPTG induction.

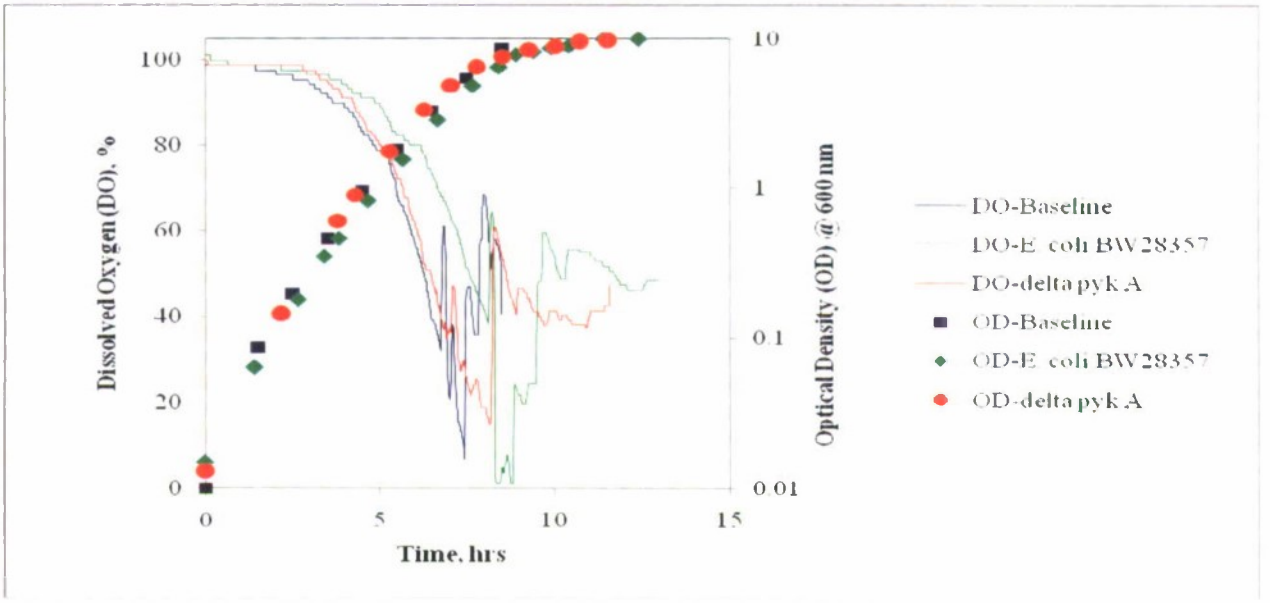


Figure 1. OD and DO Profiles during Growth of *E. coli* BW28357 with and without IPTG Induction and **Δ** pyk A mutant *E. coli* with IPTG Induction.

Time-dependent SDS-PAGE gel band intensities of the expressed P450 BM-3 proteins (MW around 150 Kdal) during 20-L fermentation runs with *E. coli* BW28357 and Δ pyk A mutant *E. coli* hosts are shown in Figures 2 and 3, respectively, as a function of elapsed fermentation time (EFT).

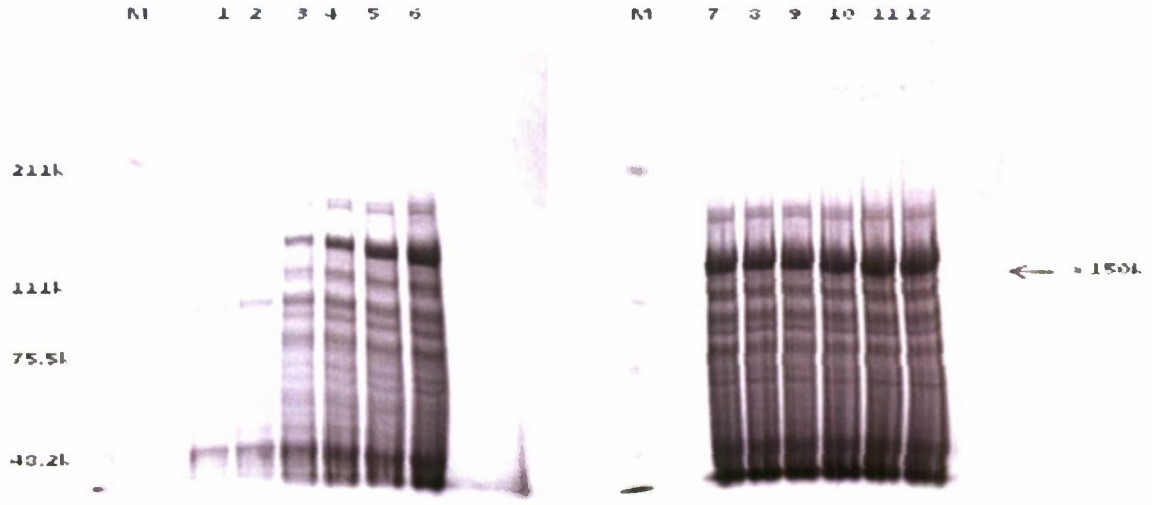

Figure 2. SDS-PAGE Gels of *E. coli* BW28357 20-L Run: M = MW markers, 1 = 3.84 hr, 2 = 4.67 hr (Induced at this time), 3 = 5.67 hr, 4 = 6.67 hr, 5 = 7.67 hr, 6 = 8.42 hr, 7 = 8.92 hr, 8 = 9.42 hr, 9 = 9.92 hr, 10 = 10.42 hr, 11 = 11.42 hr, and 12= 12.42 hr.

As illustrated in Figure 4, immediately following the IPTG induction, the level of expressed P450 BM-3 increased rapidly. However, when propane was introduced, the level dipped and recovered, for unknown reasons, to a final level of approximately 30% of the total cellular proteins, resulting in a reasonably high level of expression.

The corresponding production levels of propanol in *E. coli* BW28357 and Δ pyk A mutant *E. coli* are shown in Figure 5. The rates of propanol production in the *E. coli* BW28357 and Δ pyk A mutant *E. coli* 20-L fermentation runs were similar except for the final propanol concentration in the Δ pyk A mutant *E. coli* run that was slightly lower. However, within the experimental accuracy, both runs resulted in a propanol concentration level of 2 mM.

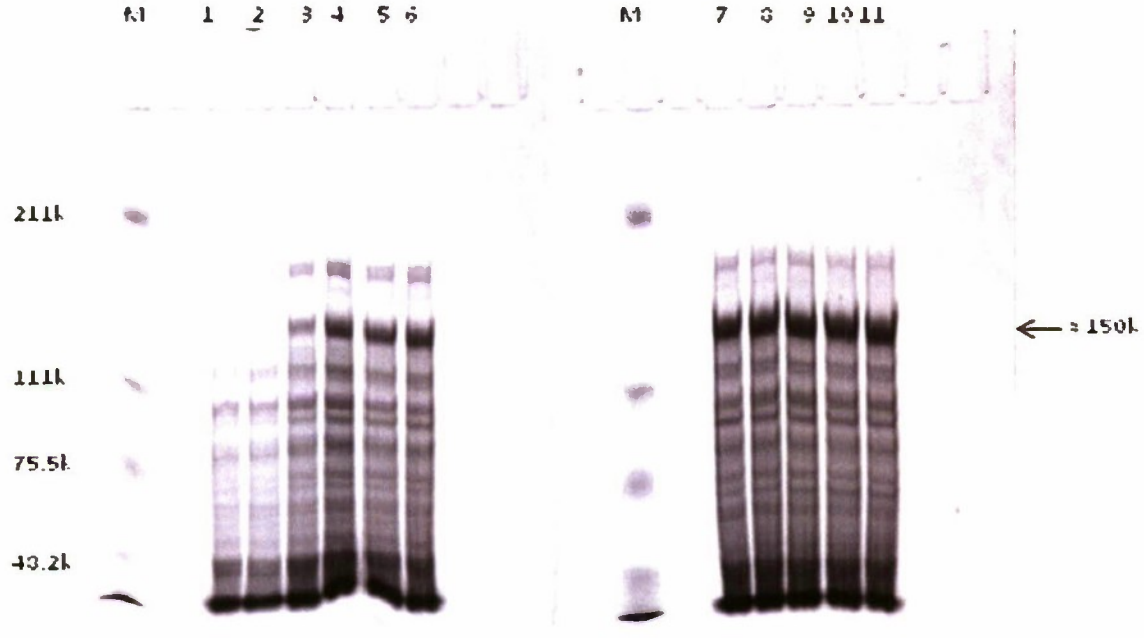


Figure 3. SDS-PAGE Gels of Δ pyk A mutant *E. coli* 20-L Run: M = MW markers, 1 = 3.78 hr, 2 = 4.28 hr (Induced at this time), 3 = 5.28 hr, 4 = 6.28 hr, 5 = 7.03 hr, 6 = 7.78 hr, 7 = 8.53 hr, 8 = 9.28 hr, 9 = 10.03 hr, 10 = 10.78 hr, and 11 = 11.53 hr.

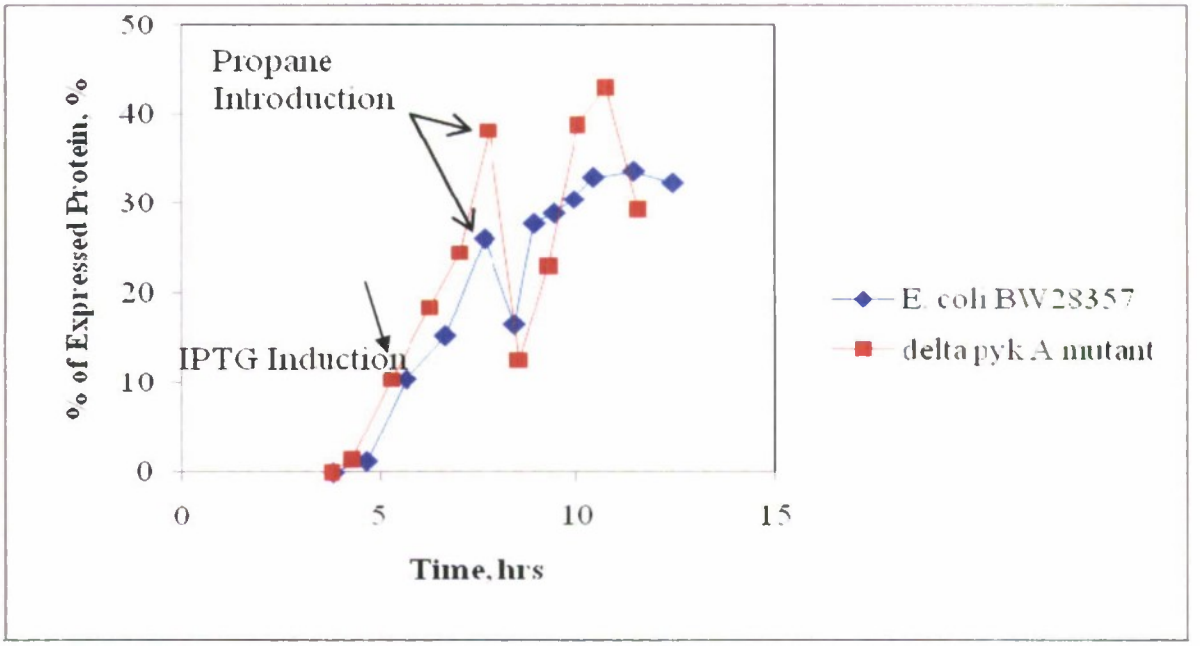


Figure 4. Time-dependent Level of Expressed P450 BM-3 as Percentage of Total Cellular Proteins.

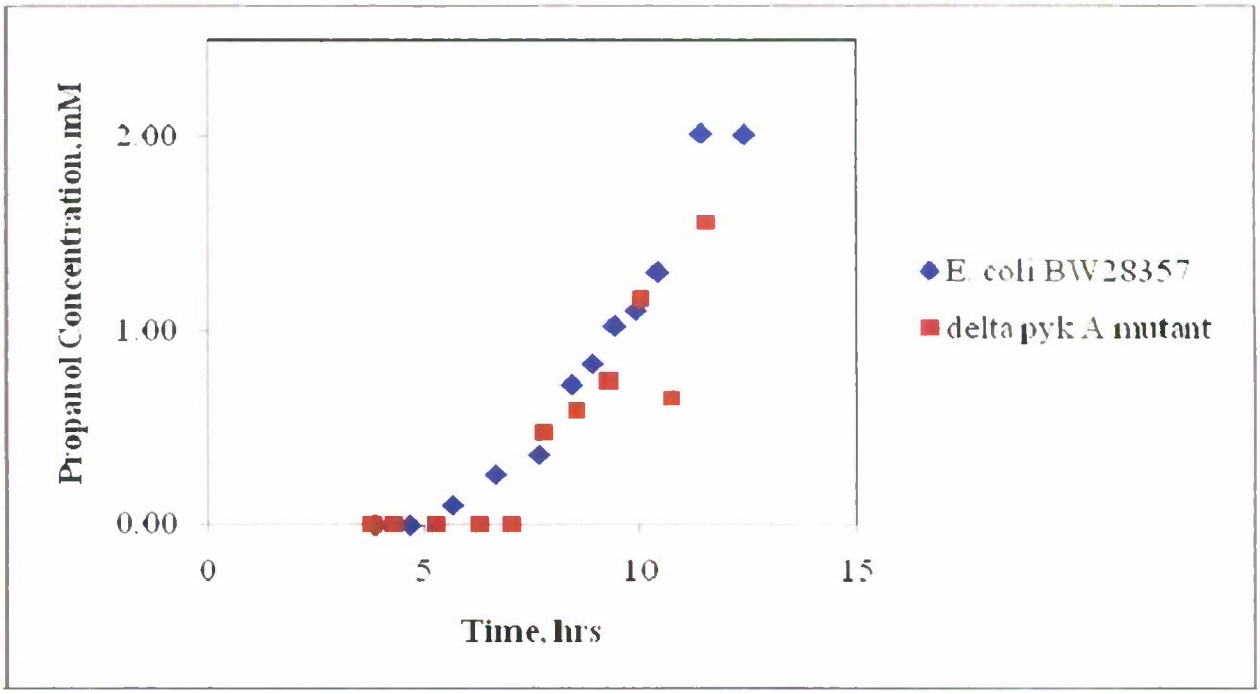


Figure 5. Propanol Concentration Level in Culture Medium.

The hypothesis that the Δ pyk A mutant *E. coli* host would siphon-off more of reducing power (i.e., NADPH) for the NADPH-dependent biotransformation of propane to propanol did not result in an improved yield of propanol. In addition, the final propanol concentration of 2 mM after 4.75 and 4.5 hr, respectively, for *E. coli* BW28357 and Δ pyk A mutant *E. coli* 20-L runs of continuous feed of propane at 10 slpm yielded an overall mass balance of only 0.0017% of the total fed propane being biotransformed to propanol. Both findings indicate that the main obstacle of the process may be a result of a low catalytic turnover rate of the expressed P450 BM-3 enzymes, despite the level of expression (e.g., approximately 30% of the total proteins) for both *E. coli* hosts.

## 4. CONCLUSIONS

Two *E. coli* hosts harboring the same PMO plasmid, one with pyk A mutation and one without, were used in a 20-L scale up fermentation process to compare the effect of pyk A mutation in propane to propanol biotransformation yield. No significant improvement or difference in yield (both approximately 2 mM) was obtained. In addition, the overall propane mass balance indicated that only 0.0017% of the total propane fed to the 20-L fermentor was biotransformed to propanol. Both results indicate a low catalytic turnover rate of the expressed P450 BM-3 enzymes.

The idea of biotransforming alkane to its corresponding alcohol appears novel. However, before it can be practically scaled-up for a production of alcohol, there seem to be many obstacles and challenges, especially in improving substrate (i.e., methane) binding and catalytic turnover rate. Other limiting factors for the scale-up considerations may include that of decoupling of the NADPH-dependency of the expressed enzymes and low solubility of the gaseous alkane substrate. Most of the gaseous substrate exits the biotransformation process, which would require a closed system to recycle and reuse gaseous substrate in the system.

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